UDC 575:630
DOI: 10.2298/GENSR1501185Z
Original scientific paper

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF UNUSUAL X-TYPE HMW GLUTENIN SUBUNITS FROM $1S^L$ GENOME OF *Aegilops longissima*

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Zhu G., S. Wang, S. Zhen, X. Shen, S. Prodanovic, and Y. Yan (2015*): Molecular characterization and phylogenetic analysis of unusual x-type HMW glutenin subunits from 1S^l genome of Aegilops longissima-* Genetika, Vol 47, No 1, 185-203.

Wheat related diploid species *Ae. longissima* (2n=2x=14, S^lS^l) has extensive storage protein variations that may provide useful gene resources for wheat quality improvement. In this work, five novel $1S^l$-encoded x-type high molecular glutenin subunits (HMW-GS) were identified and designated as $1S^l$x-123, $1S^l$x-129, $1S^l$x1-136, $1S^l$x2-136 and $1S^l$x2.2, respectively. Their complete open reading frames (ORFs) were cloned and sequenced by AS-PCR, which contained 2874 bp (956 aa) for $1S^l$x-123, 2946 bp (979 aa) for $1S^l$x-129, 2901 bp (965 aa) for $1S^l$x1-136, 2982bp (991 aa) for $1S^l$x2-136 and 2928 bp (974 aa) for $1S^l$x2.2. Molecular characterization demonstrated that five unusual subunits had greater repetitive domains resulted from a larger fragment insertion (74-113 aa). Particularly, $1S^l$x-129 had an extra cysteine residue at the position 109 due to a TAT → TGT dot mutation, which may improve the formation of superior gluten macropolymer. Our results suggest that these unusual HMW-GS could be served as potential superior gene resources for improving wheat gluten quality. Phylogenetic analysis revealed that HMW-GS genes from *Glu-1Sx* genomes had close evolutionary relationships with those of *Glu-1Dx* genome while sequences from *Ae. speltoides* aligned

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with those of B genome.

Key words: Ae. longissima, HMW-GS, molecular clone, phylogenetics

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is one of the three main cereal crops in the world and provides a major source of energy and nutrition for human. Since the past few decades, its structures and properties have been widely studied because of its significant values. With the global population and economic trends, increasing wheat production and enhancing grain quality are vitally important (TILMAN *et al*., 2002; PFEIFER *et al*., 2014). Gluten proteins account for 80-85% of total flour proteins and play vital roles in physical and functional properties of dough (WEEGELS *et al*., 1996). They are typically divided into two fractions: the monomeric gliadins providing viscous flow and extensibility to the dough and the polymeric glutenins determining the dough viscoelasticity (PAYNE, 1987; PAYNE *et al*., 1988). Glutenins compose of high and low molecular weight glutenin subunits (HMW-GS and LMW-GS). Although HMW-GS only account for about 5-10% of the total flour proteins, they are particularly important in conferring dough strength and bread-making quality (PAYNE *et al*., 1988; MA *et al*., 2005).

HMW-GS are encoded by *Glu-A1, Glu-B1* and *Glu-D1* on the long arms of the homologous group-1 chromosomes in bread wheat and each locus consists of two tightly linked genes, named x- and y-type genes according to their sizes and sequences of conserved N-terminal domains (PAYNE and LAWRENCE, 1983; HALFORD *et al*., 1987). Previous studies showed that the primary structure of mature HMW-GS consists of a conserved N- and C-terminal region and a variable central repetitive domain (GUO *et al*., 2010). The repetitive domains in x-type HMW-GS are mainly composed of repeat motifs of tripeptide, hexapeptide and nonapeptide, while there were only hexapeptide and nonapeptide in y-type HMW-GS. The differences among various HMW-GS are mainly resulted from repeat motif variants in central repetitive domains (GIANIBELLI *et al*., 2001). Meanwhile, the cysteine residues, forming intra- and inter-chain disulphide bonds, play important roles in the structure and function of gluten, whose numbers and locations are usually conserved in either x- or y-type of HMW-GS (LI *et al*., 2004; PANG and ZHANG, 2008). It is generally accepted that HMW-GS with extra cysteines, longer regularly repetitive domains and higher expression amount benefit to form superior gluten macropolymer and good bread-making quality (TAMÁS *et al*., 2002; SHEWRY *et al*., 2003; RAGUPATHY *et al*., 2008).

In the past several decades, the allelic variations at *Glu-1* loci in bread wheat have been widely investigated (RASHEED *et al*., 2014). PAYNE and LAWRENCE (1983) firstly reported the nomenclature and catalog of HMW-GS alleles identified in about 300 bread wheat cultivars, including 3 at *Glu-A1*, 11 at *Glu-B1* and 6 at *Glu-D1*. Among these HMW-GS identified, only a few ones were found to be superior quality subunits such as Dx5+Dy10 (PAYNE *et al*., 1987), By8 (YAN *et al*., 2009) and Ax1 (HALFORD *et al*., 1992). This indicates that the allelic variations at *Glu-1* loci of bread wheat are limited. However, wheat-related species as important genetic resources such as *Aegilops, Agropyrum, Eremopyrum, Secale* and *Thinopyrum*, has more extensive storage protein variations that may contribute to the improvement of bread-making quality (RODRÍGUEZ-QUIJANO *et al*., 2001; WANG *et al*., 2012). Compared with bread wheat, there are quite a few HMW-GS from related species owning an unusual number and/or location of cysteine residues (CAO *et al*., 2014). Thus, it is highly important to explore new HMW-GS gene resources among wheat related species that have potential value for gluten quality improvement.

It is widely accepted that the S genome is the progenitor of the B genome. The section

Sitopsis of *Aegilops* genus includes *Ae. bicornis* (S^b), *Ae. longissima* (S^l), *Ae. searsii* (S^s), *Ae. sharonensis* (S^{sh}), and *Ae. speltoides* (S), among which they are closely related based on cytogenetic and molecular genetic investigations (SASANUMA *et al*., 2004; YEN *et al*., 1990; ZHANG *et al*., 2001). In particular, *Ae. longissima* was found to have extraordinary features such as eyespot and pre-harvest sprouting resistance (SHENG *et al*., 2012; SINGH *et al.*, 2013), and superior rheological property (WANG *et al*., 2013). For the HMW-GS variations in *Ae. longissima*, some studies showed the potentials of the S^l-genome encoded HMW-GS in wheat quality improvement (JIANG *et al*., 2012; WANG *et al*., 2013; GARG *et al*., 2014).

In this work, we identified five new unusual x-type HMW-GS from $1S^l$ genome of *Aegilops longissima* and isolated their complete encoding genes. Their molecular structure features, heterologous expression and phylogenetic relationships with those from other *Aegilops* and *Triticum* species were investigated. Our results demonstrated that these HMW-GS are expected to be used as new gene resources for wheat quality improvement.

MATERIALS AND METHODS

Plant materials

Three accessions (PI604123, PI604129 and PI604136) from *Ae. longissima* (2n=2x=14, S^lS^l) and an addition line CS+$1S^lS^l$ (*T. aestivum* var. Chinese Spring, AABBDD+$1S^lS^l$) kindly provided by Dr. S. Hsam from Plant Breeding Institute, Technical University of Munich, Germany, were used as materials in the work. Chinese Spring (CS) with HMW-GS (null, 1Bx7+1By8, 1Dx2+1Dy12), MG7249 (1Ax2*, 1Bx7+1By8, 1Dx2.2+1Dy12) and CS substitution line CS-$1S^l$(1B) (null, $1S^l$x2.3*+S^ly16*, 1Dx2+1Dy12) (WANG *et al*., 2013) were used as references for HMW-GS identification.

Glutenin extraction and SDS-PAGE

HMW-GS were extracted from crushed mature seeds and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on YAN *et al*. (2003) and the gel was operated with 15 mA/gel in 1.5 hours then stained with Coomassie Brilliant Blue R-250 and destained with the mixture of ethanol and acetic acid.

LC-MS/MS

The peptide sequences of the native seed HMW glutenin subunits were identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). The specific HMW-GS band on the SDS-PAGE gel was excised and digested with trypsin according to JIN *et al*. (2012). A sample of the digested protein (0.5 ml) was subject to MS analysis in Waters SYNAPT HDMS mass spectrometer. The ProteinLynx Global SERVER™ (PLGS) was used to analyse the LC-MS/MS data.

Chromosome observation and counting

To confirm chromosome ploidy, the procedures of chromosome observation are as followings: (1) plump seeds were selected to culture in petridish with filter paper; (2) 0.5-1 cm root tips were clipped to be soaked in colchicine for 3 hours; (3) the root tips were added to Carnoy to fix for 24 hours, then putted in 90%, 80% and 70% alcohol in order. The tips treated were stored in 70% alcohol until used; (4) after watering root tips, they were putted in dissociative fluid for 10 min, then putted on slide after rinsing using modified phenol fuchsine dye for 10 min;

(5) the slice was observed in optical microscope and clear image was stored to count chromosome numbers.

DNA extraction, AS-PCR cloning and sequencing

Genomic DNA was extracted from fresh leaves with CTAB protocol based on YAN *et al.* (2004). A pair of allelic-specific (AS) PCR primers Sx1F (CCTTCACTATCTCAT CATCCCAC) and Sx1R (TAGGAGTCTGTTCGCATTCAGTGGC) were designed by Primer 5.0, and used to amplify the complete opening reading frames (ORFs) of the x-type HMW-GS. The designed primer pairs were expected to amplify the sequences including complete ORFs plus partial upstream and downstream segments. The PCR amplification included an initial denaturation step at 94°C for 5 min, followed by three procedures: denaturation at 94°C for 45s, anneal at 60°C for 1min, extension at 72°C for 80s, in total of 34 cycles, and a final extension step at 72°C for 10 min. The PCR products were separated in 1% agrose gel and the expected fragments were collected and purified by using the Gel Extraction Kit (Omega), and cloned with pGEM-T plasmid vector (Promega). Three positive clones were picked randomly and sequenced by TaKaRa Biotecnology (Dalian) CO., LTD, China.

SNPs and InDels identification

The complete coding sequences of the cloned genes and previously characterized HMW-GS genes from *Triticum* and related species were compared, and single nucleotide polymorphism (SNP) and insertion/deletion (InDel) variations were identified through sequence aligment and performed by software BioEdit (http://www.mbio.ncsu.edu/ bioedit/bioedit.html).

Prokaryotic expression and Western-blotting detection

To determine the expressed proteins of the cloned genes coinciding with those in seeds, the representative *Glu-S^{l}x* gene was selected to express in *E. coli*. The primers SxEF (AAACATATGGTGGCTCTCACCGTCGCTG) and SxER (ACCGAATTCCTAT CACTGGCTGGCCGAC) were redesigned with Primer 5.0, in which two restriction sites for *NdeI* and *EcoRI* (underlined) were added to ensure the expressed protein without signal peptide. The PCR fragment was collected, purified and ligated into pET-28a (Novagen), and the hybrid vector (pET-1S^{l}x2.2) was transformed into *E. coli* strain BL21 (DE3) plysS containing the HMW-GS gene as reported by ZHANG *et al.* (2008). The heterologous expression was induced by adding 1mM IPTG for 4-6 hours and the target protein was extracted by using 50% (v/v) i-propanol containing 1% DTT and separated by SDS-PAGE as described before. For further verifying the bacterial expression proteins, Western-blotting experiment was performed using a polyclonal antibody specific for HMW-GS based on the His-tag sequence present in the downstream of the cloned gene as reported previously by YAN *et al.* (2009).

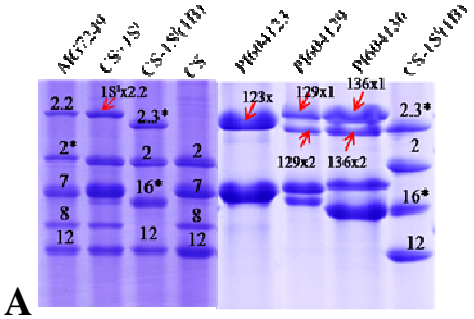
Construction of phylogenetic trees

For phylogenetic analysis, the published HMW-GS from different species were collected from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and used to perform a multiple alignment by ClustalW and MEGA 5.1.0 program. Phylogenetic trees were constructed based on complete protein sequences and conserved domain sequences excluded central repetitive domain, respectively. The related parameters of constructed phylogenetic trees were as TAMURA *et al.* (2007) and WANG *et al.* (2011).

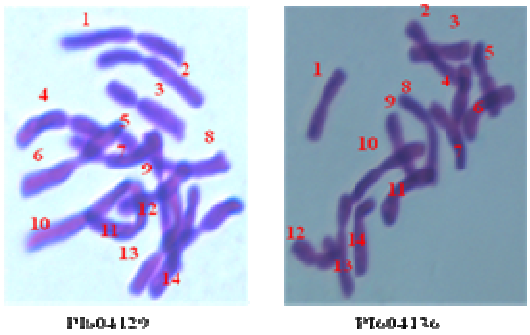
RESULTS

Identification of unusual x-type HMW-GS encoded by 1S^l genome of Ae. longissima

SDS-PAGE profiles of three *Ae. longissima* accessions and the addition line CS+1S^{l}1S^l were showed in Fig. 1A. The results showed that four materials contained six larger x-type HMW-GS and they were designed as 1S^{l}x-123 in PI604123, 1S^{l}x$_1$-129 and 1S^{l}x$_2$-129 in PI604129, 1S^{l}x$_1$-136 and 1S^{l}x$_2$-136 in PI604136, and 1S^{l}x2.2 in CS+1S^{l}1S^l. 1S^{l}x$_2$-129 and 1S^{l}x$_2$-136 had same mobility, and thus they were identified as one x-type subunit. In general, diploid species should have 1-2 HMW-GS, but both PI604129 and PI604136 had four HMW-GS. Previous study confirmed that the overexpression of 1Bx7^{OE} was resulted from gene duplicate of *Glu-B1* locus (RAGUPATHY *et al*., 2008). Therefore, we speculated that PI604129 and PI604136 could have two gene copies encoding two x-type subunits. To confirm this scenario, their chromosome numbers were observed and both contained normal 14 chromosomes as other diploid species (Fig. 1B). This suggests that one *Glu-1* locus duplicate event could occur in both accessions.



A



B

Figure 1. SDS-PAGE analysis of HMW-GS (A) and chromosome identification in *Aegilops longissima* accessions (B). The unusual x-type HMW-GS are marked by red arrows. The chromosome numbers of PI604129 and PI604136 are clearly visible. A total of 14 chromosomes indicate that both PI604129 and PI604136 are diploid, suggesting one duplication event at *Glu-1* locus in the 1S^l genome could occur.

LC-MS/MS was performed to further identify the five unusual x-type subunits separated by SDS-PAGE after digested by trypsin. The identified peptide sequences were summarized in Suppl. Table 1, which showed that all subunits belonged to HMW-GS from *Aegilops* genus.

Molecular characterization of unusual HMW-GS encoding genes from 1S^l genome

The encoding genes of five unusual x-type HMW-GS were amplified and cloned using Sx1F and Sx1R primers, which were designated as *1S^{l}x-123* from PI604123, *1S^{l}x-129* from PI604129, *1S^{l}x$_1$-136* from PI604136, *1S^{l}x$_2$-136* from PI604136 and *1S^{l}x2.2* from CS+1S^{1}S^1. According to the DNA sequencing results, the lengths of ORFs of five genes were 2928 bp for *1S^{l}x-123*, 2874 bp for *1S^{l}x-129*, 2946 bp for *1S^{l}x$_1$-136*, 2901 bp for *1S^{l}x$_2$-136* and 2982 bp for *1S^{l}x2.2*, which encoded 974, 956, 980, 965 and 992 amino acid (aa) residues, respectively (Table 1). Five new gene sequences were deposited in GenBank with the accession numbers from KF880596 to KF880600.

Table 1. The structural features of five unusual HMW-GS encoded by 1S^l genome

Subunits	Ms	N-ter		RD		C-ter		Whole protein		
		Res[a]	Cys[b]	Res[a]	Cys[b]	Res[a]	Cys[b]	Res[a]	Cys[b]	PI
1S^{l}x-123	**99503**	86	3	807	0	42	1	**935**	4	5.23
1S^{l}x-129	**101726**	86	3	831	**1**	42	1	**959**	**5**	5.55
1S^{l}x1-136	**103352**	86	3	843	0	42	1	**971**	4	6.24
1S^{l}x2-136	**100465**	86	3	816	0	42	1	**944**	4	6.59
1S^{l}x2.2	**101433**	86	3	825	0	42	1	**953**	4	5.39
1Slx2.3*	**97851**	86	3	792	0	42	1	**920**	4	6.50
1Dx2.2	**103048**	89	3	819	0	42	1	**950**	4	5.24
1Dx2	87004	88	3	687	0	42	1	817	4	5.39
1Bx13	83206	81	3	651	0	42	1	774	4	**8.89**
1Bx14	84012	86	3	646	0	42	1	774	4	**9.16**

Ms, N-ter, RD, C-ter and PI represent Molecular mass, N-terminal domain, Repetitive domain, C-terminal domain and isoelectric point, respectively. a. The number of residues (Res). b. The number of cysteine residues (Cys).

The nucleotide lengths of three new cloned genes (*1S^{l}x-129*, *1S^{l}x$_1$-136* and *1S^{l}x2.2*) are longer than previously characterized *1Dx2.2* gene (WAN *et al*., 2005), the second largest subunit present in bread wheat (only 159 bp shorter than *1Dx2.2**). The lengths of *1S^{l}x-123* and *1S^{l}x$_2$-136* are 45 bp and 18 bp shorter than that of *1Dx2.2*, respectively. However, all five new genes are longer than recent cloned *1S^{l}x2.3** gene (2928 bp) from 1S^l genome, which was considered as the largest subunit among 1B genome-encoded HMW-GS in bread wheat (WANG *et al*., 2013).

Analysis of the deduced amino acid sequences of five cloned genes showed that the lengths of signal peptide, N- and C-terminal domains are 21, 86 and 42 aa, respectively, and all belonged to typical x-type HMW-GS (Table 1; Fig. 2-3). The number and position of cysteine residues were highly conserved: three in N-ter and one in C-ter. But in 1S^{l}x-129, an extra cysteine was present at the position 109 due to a TAT → TGT dot mutation (Fig. 2). In addition, the isoionic points (PI) of five mature proteins were calculated by ExPASy site

(http://www.expasy.org/). Obviously, the PI of the 1B genome-encoded subunits is higher than that of the $1S^l$ and 1D genome-encoded subunits, suggesting that the protein subunits encoded by the B genome appear to be basic while those from the $1S^l$ and 1D genomes tend to be acidic (Table 1).

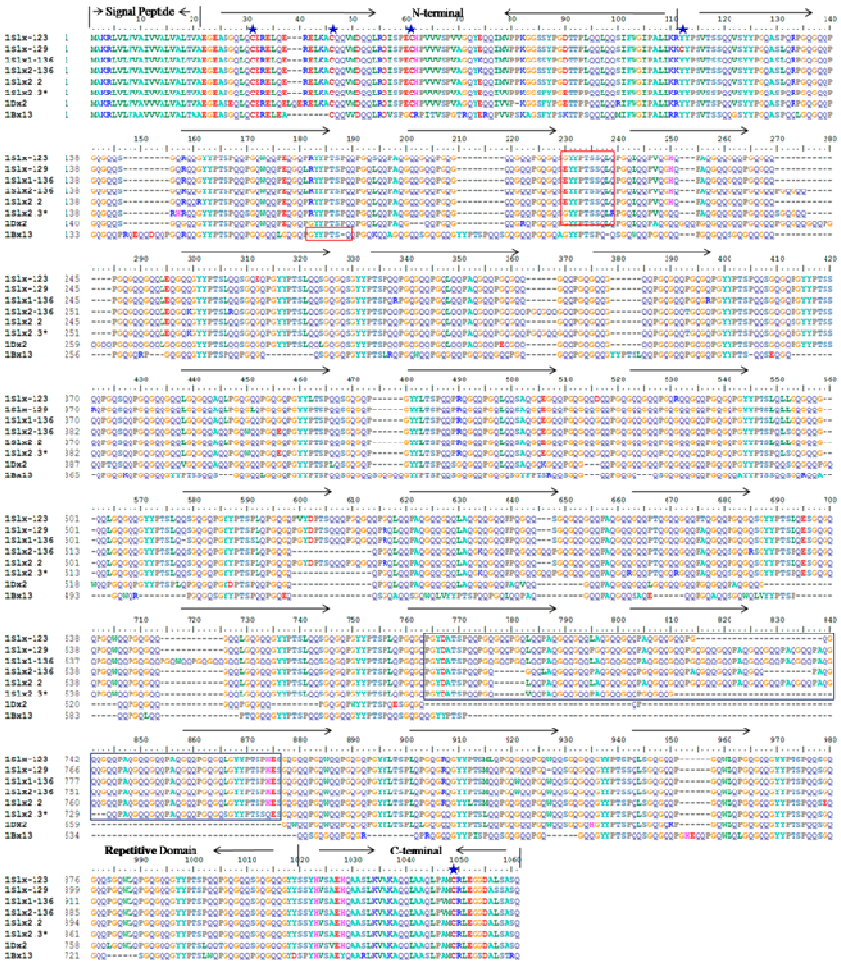


Figure 2. Multiple alignment of x-type HMW-GS from $1S^l$, B and D genomes. Four conserved domains were indicated: Signal Peptide, N-terminal, Repetitive Domain (RD) and C-terminal. The conserved cysteines (three in N-ter and one in C-ter) and an extra cysteine present in RD from $1S^l$x-129 were highlighted by blue asterisk. A specific ocapeptide and decapeptides were highlighted by red boxes. Larger fragments inserted were marked by blue box.

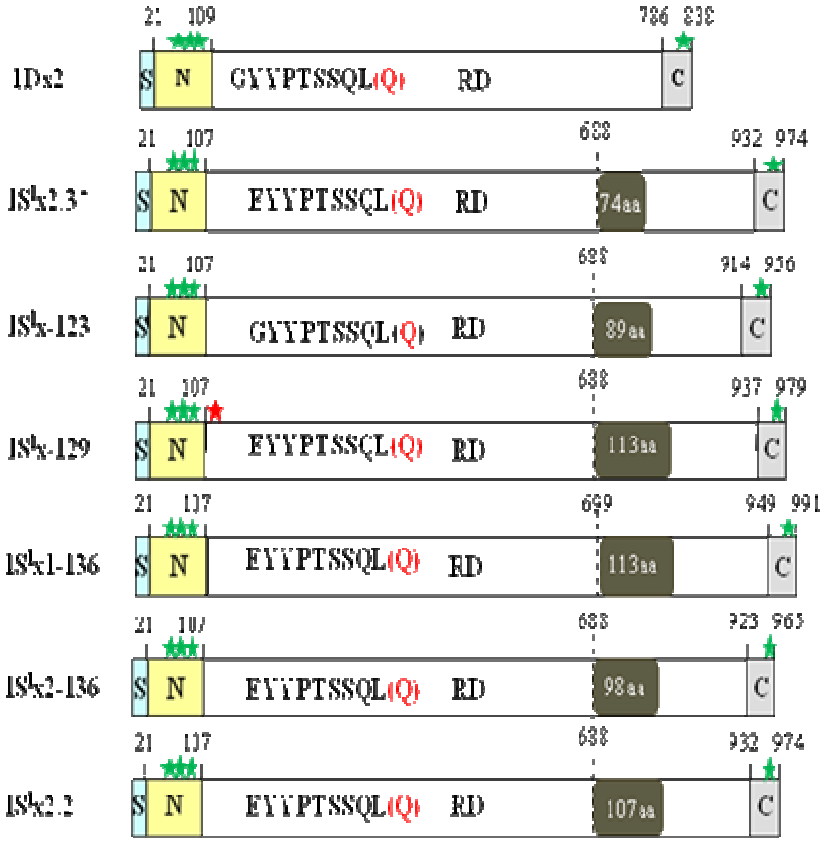


Figure 3. Schematic diagram of the primary structure of HMW-GS encoded by *Glu-D1* and *Glu-S*^{l}. S, N, RD and C represent Signal Peptide, N-terminal, Repetitive Domain and C-terminal, respectively. Conserved cysteine residues are indicated by green asterisk, while an extra cysteine residue is indicated by red asterisk. The inserted fragments are indicated by gray box and the length of each insertion is numbered in the box. The specific decapeptides in the repetitive domain are indicated.

As shown in Fig. 2, the distribution of repetitive motifs in different genomes was distinct. For example, the total numbers of tripeptide in 1Bx13 of B genome were greatly less than those in the subunits encoded by other genomes. The major hexapeptide consensus the 1S^{l} genome encoded subunits was PGQGQQ and PAQGQQ, while a few variations were present in the 1B genome (PGQGQQ and SGQGQQ) and 1D genome (PGQGQQ). GYYPTSPQQ is staple nonapeptide among three genomes. A decapeptide was present in the subunits encoded by the 1S^{l} and 1D genome while there is an octapeptide in those from 1B genome, which were resulted from

insertion or deletion in single repeat motif. As indicated in Table 2, five unusual subunits showed higher similarity to those from 1D genome.

Table 2. The summary of repetitive peptide motifs in the repetitive domains of HMW-GS

Subunits	Tripeptide				Hexapeptide								Nonapeptide			Octapeptide	Decapeptide			
	T	Category	Total	R	H1	H2	H3	Category	Total	R	N1	N2	Category	Total	R	O	D1	D2	D3	D4
1Slx-123	21	3	24	0.375	21	9	7	26	81	19.259	5	4	14	23	9.57	_	_	1	_	_
1Slx-129	24	3	26	0.231	22	13	7	25	84	18.452	6	4	14	23	10.348	_	_	_	1	_
1Slx1-136	24	3	26	0.231	22	13	7	25	86	18.605	5	4	16	23	12.522	_	_	_	1	_
1Slx2-136	24	3	26	0.231	23	12	7	26	84	18.881	6	3	13	21	9.286	_	_	_	1	_
1Slx2.2	24	3	26	0.231	21	13	6	26	84	19.5	5	4	14	23	10.957	_	_	_	1	_
1Slx2.3*	22	3	24	0.25	23	9	8	26	81	18.617	6	5	11	22	8	_	_	_	_	1
1Bx13	**3**	2	**4**	0.5	16	3	17	30	69	23.043	12	1	10	23	4.783	1	_	_	_	_
1Dx2	17	3	19	0.315	23	6	6	21	72	14.29	6	4	9	18	6	_	_	1	_	_

T, GQQ; H1, PGQGQQ; H2, PAQGQQ; H3, SGQGQQ; N1, GYYPTSPQQ; N2, GYYPTSLQQ; O, GYYPTS_QQ; D1, GYYPISSQL(**Q**); D2, GYYPTSSQL(**Q**); D3, EYYPTSSQL(**Q**); D4, GYYPTSSQL(**R**)

R = variant motif number*category/total motif number.

Table 3. The positions of SNPs indentified among five genes cloned compared to 21 other x-type HMW-GS genes from Triticeae and related species.

HMW-GS Genes	27	232	257	270	281	339	464	709	1865	1868	1959	2248	2941	3528
1S^{l}x2.2	**A**	**A**	**C**	**C**	**T**	**T**	**A**	**T**	**T**	**T**	**G**	**A**	**T**	**T**
1S^{l}x-123	**A**	**A**	**C**	**C**	**T**	**T**	**G**	**T**	**T**	**T**	**G**	**A**	**T**	**T**
1S^{l}x-129	**A**	**A**	**C**	**C**	**T**	**T**	**G**	**T**	**T**	**T**	**G**	**A**	**T**	**T**
1S^{l}x1-136	**A**	**A**	**C**	**C**	**T**	**T**	**G**	**T**	**T**	**T**	**G**	**A**	**T**	**T**
1S^{l}x2-136	**A**	**A**	**C**	**C**	**T**	**T**	**G**	**T**	**T**	**C**	**G**	**A**	**T**	**T**
21 other x-type HMW-GS Genes[a]	G	G	T	G	C	C	G	C	A	C	A	G	C	C

21 other x-type HMW-GS genes were from GenBank, including GQ403043 (Sx, *Ae. speltoides*), AY611723 (Sx, *Ae. speltoides*), HQ380225 (Sx1*, *Ae. speltoides*), HQ380224 (Sx3*, *Ae. speltoides*), X61009 (1Ax1, *T.aestivum*), DQ533690 (GluA1-2, *T. aestivum*), M22208 (Ax2*, *T. aestivum*), JF938073 (1Bx7^{OE}, *T. aestivum*), EF540764 (1Bx13, *T. aestivum*), AF476961 (Ux, *Ae. umbellulata*), AJ893508 (1Dx2.2*, *T. aestivum*), X12928 (1Dx5, *T. aestivum*), AY455789 (Mx, *Ae. comosa*), AY455786 (Nx, *Ae. uniaristata*), AF216868 (R1, *T. Aestivum*), X03346 (1Dx2, *T. aestivum*), DQ857243 (1Dx1.6^{t}, *Ae. tauschii*), AF480485 (1Dx2^{t}, *Ae. tauschii*), HM124446 (1Dx3.1^{t}, *Ae. tauschii*), DQ478572 (H1Dx5, *Th. ponticum* x *T. aestivum*), DQ478576 (1Aex2, *L. elongatum*).

When comparing with 1Dx2, six $1S^{l}$x genome-encoded subunits shown in Fig. 3 share different degrees of insertions ranging from 74 aa to 113 aa at the same position 688, except for $1S^{l}$x1-136 at position 699, indicating that a similar duplication event occurred during the evolutionary process. Meanwhile, the complete coding sequences of the five cloned genes and 21 other HMW-GS genes from *Triticum* and related species were compared and SNP and InDel variations were identified. As shown in Table 3, a total of 70 SNPs were identified at 14 positions, of which 55 (78.6%) were resulted from transitions (A-G or C-T), while InDels were not observed. Thus, although the five genes displayed higher similarity with those from *Triticum* and related species, some allelic variations among them still existed (marked by red characters).

Heterologous expression and Western-blotting analysis of $1S^{l}$x2.2 gene

To confirm the authenticity of the cloned genes, the representative *$1S^{l}$x2.2* gene was selected for heterologous expression in *E. coli*. A pair of primers was designed to ensure the expressed protein of the cloned ORF without signal peptide, the modified ORF was ligated with pET-28a vector, and the pET-$1S^{l}$x2.2 expression vector was constructed. *E. coli* expression was induced by IPTG and the expressed protein was identified by both SDS-PAGE and Western-blotting. As shown in Fig. 4A, one overexpressed protein band in *E. coli* was identified, which had similar electrophoretic mobility with the native subunit from the seed. Western-blotting experiment using polyclonal antibody specific for HMW-GS showed that the heterologous expressed protein had a strong reaction to the polyclonal antibody, while the empty pET-28a vector had no any reaction signal (Fig. 4B). This further verified the authenticity of *$1S^{l}$x2.2* bacterial expression.

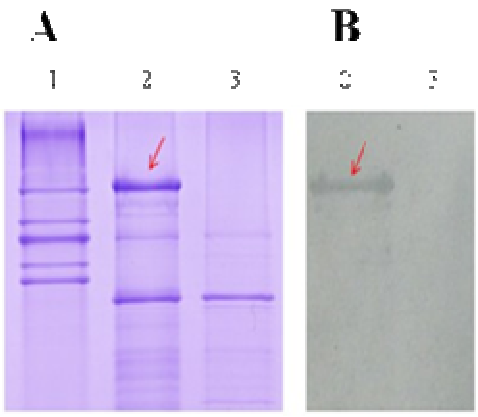


Figure 4. Heterologous expression of the modified ORFs of *$1S^{l}$x2.2* gene in *E. coli* and SDS-PAGE (A) and Western-blot (B) analyses of the expressed protein. The coding sequence for the signal peptide was removed from the cloned ORFs by PCR amplification. The modified ORFs were then used to construct with pET-28a. Line 1: Chinese addition line CS+$1S^{l}$ (2n=44, AABBDD+$1S^{l}S^{l}$); Line 2: the expressed protein of pET-$1S^{l}$x2.2; Line 3: the expressed protein arrowed of pET-28a plasmid clone.

Phylogenetic analysis of HMW-GS among common wheat and related species

To explore the evolutionary relationships of HMW-GSs between S^l genome and other related genomes, a neighbor-joining tree was constructed based on the complete protein sequences from A, B, D and S^l genome by MEGA5.10 (Fig. 5A). Since the sequences of signal peptide plus N-terminal and C-terminal domains were conserved and phylogenetically informative, another phylogenetic tree was also constructed according to the amino acid sequences without repetitive domains (Fig. 5B). Both phylogenetic trees showed similar results, in which two clearly separated branches, corresponding to upper x-type and lower y-type HMW-GS groups. Among x-type HMW-GS group, four subgroups were clustered, and those from *Glu-Sx* loci of S and related genomes (S^b, S^l, S^{sh} and S^s) showed close relationships, which were more homologous to those from *Triticum* D genome.

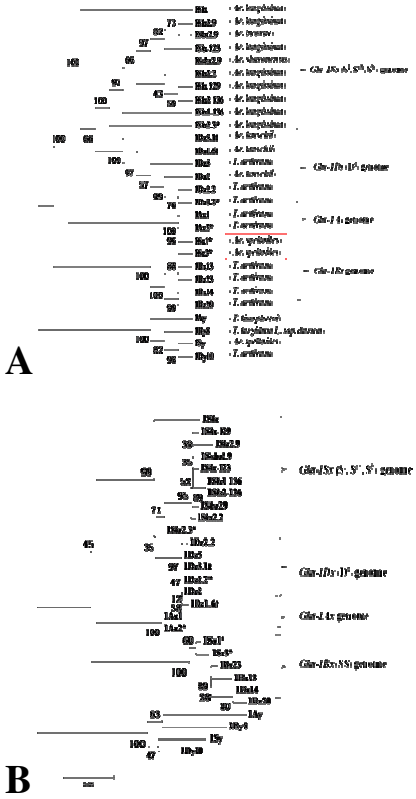


Figure 5. Phylogenentic relationships of five unusual x-type HMW-GS ($1S^lx$-123, $1S^lx$-129, $1S^lx1$-136, $1S^lx2$-136 and $1S^lx2.2$) with those from A, B, D and S genomes. The trees were constructed by MEGA5.1 software and calculated by the neighbour-joining method based on complete protein sequences (A) and the sequences removed repetitive domain (B). The bootstrap values were obtained using 1000 replications.

DISCUSSION

HMW-GS have been widely investigated in past several decades due to their important roles in determining wheat dough strength and breadmaking quality. However, variations at the *Glu-1* loci in bread wheat have shown to be limited (PAYNE and LAWRENCE, 1983). Particularly, only a few subunits encoded by *Glu-1* loci showed to have positive effects on gluten quality such as Dx5+Dy10, Ax1 and Bx17+By18 (GIANIBELLI *et al*., 2001). Compared with common wheat, wild *Triticeae* related species have more extensive HMW-GS variants (JIANG *et al*., 2012). Meanwhile, S genome of *Aegilops* genus is accepted as the wheat genome donor, and therefore, considerable work has focused on finding the potential gene resources in *Aegilops* genus (LIU *et al*., 2003; SUN *et al*., 2006; JIANG *et al*., 2012). *Ae. longissima*, as one of five species in the section *Sitopsis*, is attached more and more attention. The recent report has demonstrated that HMW glutenin subunits from $1S^l$ genome of *Aegilops longissima* can significantly improve wheat breadmaking quality (WANG *et al*., 2013). However, discovery of the potential HMW-GS variations related to superior gluten quality in *Ae. longissima* species is still limited.

In the current work, we isolated and characterized five unusual x-type HMW-GS genes from $1S^l$ genome of three *Ae. longissima* accessions and one Chinese Spring $1S^l$ addition line. Their deduced amino acid sequences contained more repetitive domains that resulted in larger molecular masses (Fig. 1-2; Table 1). In particular, $1S^l$x-129 contained an extra cysteine present at the position 109 resulting from a TAT → TGT dot mutation (Fig. 2). Meanwhile, our results from sequence alignment (Fig. 2) and phylogenetic trees (Fig. 5A, B) revealed that *Glu-1Sx* (S^l, S^{sh}, S^b, S^s) genome-encoded HMW-GS genes had close evolutionary relationships with those of *Glu-1Dx* genome while sequences from *Ae. speltoides* aligned with those of B genome, which is consistent with the current study (HAIDER *et al*., 2012; JIANG *et al*., 2012; GARG *et al*., 2014), indicating that their HMW-GS encoding genes may evolve differently.

Previous reports showed that the subunit 1Dx2.2 is considered as the second largest HMW-GS, only next to $1Dx2.2^*$ (WAN *et al*., 2005). However, five subunits characterized in this work contained 935-971 aa residues, only slightly shorter, if not longer than 950 residues of 1Dx2.2 (Table 1). This suggests that the cloned five new genes are larger than most x-type HMW glutenin subunit genes characterized so far, which may be resulted from unequal crossing over of repetitive domains. Actually, it have been reported that unequal crossover is most likely mechanism of variations in HMW-GS, i.e. duplication or deletion of large fragments in *Glu-1-1* alleles (ZHANG *et al*., 2008; JIANG *et al*., 2012), and unequal crossover between the *Glu-1* loci located on the sister chromatids of the homologous chromosomes (SUN *et al.*, 2006). In this work, there are duplications with different degrees in six $1S^l$-genome encoded subunits, compared to 1Dx2 (Fig. 3), which may be the results of unequal crossover during duplications of repetitive motifs. Furthermore, intrastrand illegitimate recombination may also result in a direct repeats deletion and generation of novel gene (LI *et al*., 2008; WANG *et al*., 2011). In addition, SNP variations caused by dot mutation may lead to nonsynonymous SNP and various amino acid substitutions, resulting in allelic variations, which may contribute to further understanding the origin and evolution of HMW-GS (LI *et al*., 2008). Based on described above, we speculate that the evolution of glutenin genes may be generated by mulitiple factors, such as dot mutation, insertions/deletions and/or duplications.

It is known that the length variations of HMW-GS are mainly determined by the size of the repetitive domain (WANG *et al*., 2013). Longer repetitive domains tend to form the more stable interaction among gluten polymers by inter-chain hydrogen bonds, which is rich in glutamine

(FEENEY *et al*., 2003; REN *et al*., 2007). For example, the 1Dx2.2* and 1Dx2.2 subunits can result in dough strength greater than the lower subunit 1Dx2. The five x-type subunits encoded by $1S^l$ genome are all larger than 1Dx2, and $1S^l$x2.2, $1S^l$x-129 and $1S^l x_2$-136 are even larger than 1Dx2.2. Therefore, the longer repetitive domains could facilitate the formation of superior gluten macropolymer. Meanwhile, according to the previous research, the consensus of tripeptide and hexapeptide in repetitive domains has certain effect on wheat quality (MASCI *et al*., 2000). The proportion of tripeptide and hexapeptide motifs in their repetitive domain is higher than that of 1Bx13 and 1Dx2 (Table 2). Compared with 1Bx13, the S^l-genome encoded subunits contain more regular hexapeptide motifs, which may contribute to produce more regular patterns of repetitive β-turns (MA *et al*., 2005). In addition, an extra cysteine residue present in 1Dx5 is considered to have positive impacts on wheat quality whereas the two cysteine residues absent in 1Bx20 lead to poor flour quality (SHEWRY *et al*., 2003; ZHANG *et al*., 2008). This indicates that the number and distribution of cysteine residues play important roles in the formations of gluten polymers. Our results found that the subunit $1S^l$x-129 contains an extra cysteine residue at the position of 109 in repetitive domain, which may have similar impacts on wheat quality as that in 1Dx5.

CONCLUSION

We isolated and characterized five unusual $1S^l$ genome-encoded HMW glutenin subunits from three *Ae. longissima* accessions and a CS addition line. A big fragment insertion in the repetitive domain made them to be unusual high molecular weight gluten subunits. Furthermore, an extra cysteine residue in $1S^l$x-129 may promote the formation of gluten polymers. Thus, these subunits can be served as potentially superior gene resources for wheat quality improvement. In addition, phylogenetic analysis revealed that HMW-GS genes from S genomes of *Sitopsis* species have closer relationships with those from *Triticum* D genome.

ACKNOWLEDGEMENT

This research was financially supported by grants from the National Natural Science Foundation of China (31271703, 31471485), Natural Science Foundation of Beijing City and the Key Developmental Project of Science and Technology, Beijing Municipal Commission of Education (KZ201410028031), and the National Key Project for Transgenic Crops of China (2011ZX08009-003-004) and Ministry of Education, Science and Technological Development, Republic of Serbia, project TR-31069.

Received October 21th, 2014
Accepted February 2 5th, 2015

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MOLEKULARNA KARAKTERIZACIJA I FILOGENETSKA ANALIZA NEOBIČNIH X-TIPOVA HMW GLUTENINSKIH PODJEDINICA IZ 1S^{L} GENOMA *Aegilops longissima*

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Izvod

Srodnik pšenice, diploidna vrsta *Ae. longissima* (2n=2x=14, S^{l}S^{l}) karakteriše se izraženom varijabilnošću rezervnih proteina i može da predstavlja koristan izvor gena za popravku kvaliteta pšenice. U ovom radu, identifikovano je pet novih 1S^{1}-kodiranih x-tip visoko-molekularnih gluteninskih subjedinica (HMW-GS) koje su označene kao 1S^{l}x-123, 1S^{l}x-129, 1S^{l}x1-136, 1S^{l}x2-136 i 1S^{l}x2.2. Njihovi kompletni otvoreni okviri čitanja (open reading frames, ORFs) bili su klonirani i sekvencionirani uz pomoć AS-PCR, a sastojali su se od 2874 bp (956 aa) kod 1S^{1}x-123, 2946 bp (979 aa) kod 1S^{l}x-129, 2901 bp (965 aa) kod 1S^{l}x1-136, 2982bp (991 aa) kod 1S^{l}x2-136 i 2928 bp (974 aa) kod 1S^{l}x2.2. Molekularna karakterizacija je pokazala da su pet neobičnih podjedinica imale veće repetitivne domene koji su posledica insercije većeg fragmenta (74-113 aa). Ističe se 1S^{l}x-129 koji je imao poseban cisteinski ostatak na poziciji 109 usled TAT→ TGT tačkaste mutacije, što može da poboljša formiranje superiornog glutenskog makropolimera. Naši rezultati ukazuju na to da HMW-GS geni mogu da posluže kao potencijalni izvori superiornih gena za poboljšanje kvaliteta glutena kod pšenice. Filogenetska analiza je pokazala da HMW-GS geni sa *Glu-1Sx* genoma su imali bliske evolucione veze sa onima sa *Glu-1Dx* genoma, dok sekvence sa *Ae. speltoides* su se podudarale sa onima sa B genoma.

Primljeno 21. X. 2014.
Odobreno 25 II. 2015.

Suppl. Table 1a. List of Protein/Peptide sequences by LC-MS/MS analysis and searching the NCBI-nr database using MASCOT

*asterisk represents M oxidized

Bands	Identified Name	Protein name	Species	Unique PepCount No.	PepCount Sequence (s)	Cover Percent	Peptide Position
1S^{l}x2.2	gi|83752437	HMW-GS: 1Dx4^t	*Aegilops tauschii*	4	33	7.42%	
					K.ACQQVM*DQQLR.D		41-53
					K.AQQLAAQLPAM*CR.L		950-964
					R.DISPECHPVVVSPVAGQYEQQIM*VPPK.G		52-80
					R.LEGGDALSASQ		963-974
1S^{l}x-123	gi|317573327	HMW-GS: Ux2.3	*Aegilops kotschyi*	8	57	10.12%	
					K.ACQQVMDQQLR.D		41-53
					K.AQQLAAQLPAM*SR.L		932-946
					K.RYYPSVTSSQQVSYYPGQASPQR.P		107-131
					R.LEGGDALSASQ.		945-956
					R.PGQGQQPGQGQQSGQR.Q		130-147
					R.QQGYYPTSPQQPGQWQQPEQGQPR.Y		146-171
					R.YYPSVTSSQQVSYYPGQASPQR.P		108-131
					R.YYPSVTSSQQVSYYPGQASPQRPGQGQQPGQGQQSGQ R.Q		108-147
1S^{l}x1-129	gi|317573327	HMW-GS: Ux2.3	*Aegilops kotschyi*	5	26	8.68%	
					K.ACQQVMDQQLR.D		41-53
					R.LEGGDALSASQ.		969-980
					R.PGQGQQPGQGQQSGQR.Q		130-147
					R.QQGYYPTSPQQPGQWQQPEQGQPR.Y		146-171
					R.YYPSVTSSQQVSYYPGQASPQRPGQGQQPGQGQQSGQ R.Q		108-147
1S^{l}x2-129	gi|317573327	HMW-GS: Ux2.3	*Aegilops kotschyi*	4	12	6.4%	Un-cloned
					K.ACQQVMDQQLR.D		
					R.LEGGDALSASQ.		
					R.PGQGQQPGQGQQSGQR.Q		
					R.QQGYYPTSPQQPGQWQQPEQGQPR.Y		

Suppl. Table 1b. List of Protein/Peptide sequences by LC-MS/MS analysis and searching the NCBI-nr database using MASCOT

*asterisk represents M oxidized

Bands	Identified Name	Protein name	Species	Unique PepCount No.	PepCount Sequence (s)	Cover Percent	Peptide Position
1S^{l}y1-129	gi|47834187	HMW-GS: 1Sy-2.5b	*Aegilops bicornis*	8	37	13.58%	Un-cloned
					K.GQQGYYPTSLQQPGQGQQIGQGQQGYYPTSPQHPGQR.Q		
					R.ELQESSLEACR.Q		
					R.LPWSTGLQM*R.C		
					R.M*EGGDALSASQ.		
					R.PVAVSQVAR.Q		
					R.QGSYYPGQASPQQPGQGQQPGK.W		
					R.QVVDQQLAGR.L		
					R.QVVDQQLAGRLPWSTGLQM*R.C		
1S^{l}y2-129	gi|47834187	HMW-GS: 1Sy-2.5b	*Aegilops bicornis*	11	51	19.38%	Un-cloned
					K.AQQPTTQLTTVCR.M		
					K.CRPVAVSQVAR.Q		
					K.GQQQGYYPTSLQQPGQGQQIGQGQQGYYPTSPQHPGQR.Q		
					K.QGYDSPYHVSAEQQAASSM*VAK.A		
					R.ELQESSLEACR.Q		
					R.LPWSTGLQM*R.C		
					R.M*EGGDALSASQ.		
					R.QGSYYPGQASPQQPGQGQQPGK.W		
					R.QVVDQQLAGR.L		
					R.QVVDQQLAGRLPWSTGLQMR.C		
					R.QYEQTVVLPK.G		
1S^{l}x2-136	gi|317573327	HMW-GS: Ux2.3	*Aegilops kotschyi*	5	29	7.75%	
					K.ACQQVM*DQQLR.D		41-53
					K.AQQLAAQLPAM*SR.L		968-982
					R.LEGGDALSASQ.		981-992
					R.PGQGQQPGQGQQSGQR.Q		130-147
					R.QQGYYPTSPQQPGQWQQPEQGQPR.Y		146-171

Suppl. Table 1c. List of Protein/Peptide sequences by LC-MS/MS analysis and searching the NCBI-nr database using MASCOT

*asterisk represents M oxidized

Bands	Identified Name	Protein name	Species	Unique PepCount No.	PepCount Sequence (s)	Cover Percent	Peptide Position
1Sy1-13	gi|317573327	HMW-GS: Ux2.3	*Aegilops kotschyi*	4	24	6.4%	
					K.ACQQVMDQQLR.D		41-53
					R.LEGGDALSASQ.		954-965
					R.PGQGQQPQGQGQSGQR.Q		130-147
					R.QQGYYPTSPQQPGQWQQPEQGQPR.Y		146-171
1Sy1-13	gi|47834187	HMW-GS: 1Sy-2.5b	*Aegilops bicornis*	8	68	16.54%	Un-cloned
					K.AQQPTTQLTTVCR.M		
					K.GQQGYYPTSLQQPGQGQQIGQGQQGYYPTSPQHPGQR.Q		
					K.QGYDSPYHVSAEQQAASSM*VAK.A		
					R.LPWSTGLQM*R.C		
					R.M*EGGDALSASQ.		
					R.PVAVSQVAR.Q		
					R.QGSYYPGQASFQQPGQGQQPGK.W		
					R.QVVDQQLAGRL.L		
1Sy1-13	gi|47834187	HMW-GS: 1Sy-2.5b	*Aegilops bicornis*	8	88	15.31%	Un-cloned
					K.AQQPTTQLTTVCR.M		
					K.GQQGYYPTSLQQPGQGQQIGQGQQGYYPTSPQHPGQR.Q		
					K.QGYDSPYHVSAEQQAASSM*VAK.A		
					R.LPWSTGLQMR.C		
					R.QGSYYPGQASFQQPGQGQQPGK.W		
					R.QVVDQQLAGRL.L		
					R.QVVDQQLAGRLPWSTGLQM*R.C		
					R.QYEQTVVLPK.G		